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BOVINE KERATOHYALIN: ANATOMICAL, HISTOCHEMICAL, ULTRASTRUCTURAL, IMMUNOLOGIC, AND BIOCHEMICAL STUDIES

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Salt extraction studies showed that keratohyalin (KH) could be solubilized and extracted from fresh bovine hoof epidermis. The solubility of KH varied in relation to the molarity of the salt solution used for extraction. Using this information, the extracted KH was aggregated in vitro by dialyzing the high salt extract against distilled water. Histochemical, ultrastructural, and immunologic studies of the resultant particles or macroaggregates showed that the latter had the same properties and immunogenicity as the KH granule in situ and produced antibodies against it. Fractionation of the macroaggregates by polyacrylamide gel electrophoresis demonstrated that the macroaggregates were composed of sets of 20 polymers whose subunits or monomers had a molecular weight of 16,900. Amino acid analyses showed that the macroaggregates and the various fractionated polymers were similar and that the protein had 116 amino acid residues. Serine, arginine, glycine, glutamic acid, and histidine constituted 78% of all residues, and serine alone represented 27%. The molecular weight by amino acid analyses was 16,150 after correction for the 8% ribonucleic acid which appears to be complexed to the protein.

This review is limited mainly to studies of bovine keratohyalin (KH), but reports on nonbovine KH will be included to establish certain correlations. The data reported here on bovine KH are, as far as I know, unique, and the isolation methods and the disc gel electrophoresis data are applicable only to the exact tissue and methods used.

In general, the review is concerned with: (1) morphologic observations of a tissue unusually rich in KH [1]; (2) salt-extraction studies of bovine KH [2]; (3) a method for isolating KH which takes advantage of its solubility properties [2–4]; (4) the histochemical and ultrastructural methods used to identify isolated KH [2,4]; (5) immunologic identification methods [5]; (6) physical-chemical characterization of isolated KH [2,4,6]; and (7) kinetic autoradiographic studies [7].

KH was reported to be highly insoluble in buffers ranging in pH from 2.6 to 8.6 as well as in 1.0 M potassium phosphate buffer (pH 7.0) [8,9]. Therefore, we attempted unsuccessfully to isolate the discrete nonmembrane-bound cytoplasmic particles by cell fractionation and differential centrifugation. Many of these experiments resulted in the puzzling observation that no KH material was found in any of the many fractions collected for ultrastructural and histochemical screening. We missed the significance of this observation and hypothesized incorrectly that the absence of KH in these fractions was due to the small amount of KH in the rat epidermis and cattle snout being studied.

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CATTLE HOOF EPIDERMIS

During this period, we began to study various bovine tissues and noticed that the epidermis of the posterior hoof contained stratum granulosum which averaged 30 cells thick [1]. Furthermore, the anatomy of the tissue was such that the viable epidermis could be dissected out with a razor blade to yield fresh nonchemically treated tissue extremely rich in KH (Fig. 1). This was a key observation because this method, in which a single molecular species of KH can be isolated, works only with ungulate hoof epidermis. We assume that this is simply due to the large amount of KH in hoof epidermis, not to any biochemical differences in the KH found in other tissues or animals.

SOLUBILITY

We began to use bovine hoof epidermis for our cell fractionation and differential centrifugation studies but again met with failure. Analyzing these

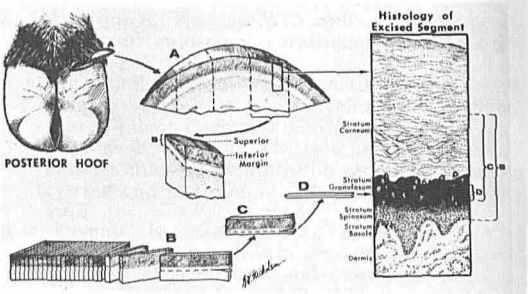


FIG. 1. The method used to excise fresh bovine epidermis.

failures, we saw a loose correlation between the salt concentration in the homogenizing solution and the absence of KH in the various fractions. To determine whether KH was as highly insoluble as had been reported, we made cryostat sections of bovine or rat skin [2,4,10], incubated them in various solutions, and histochemically monitored the extraction of KH. A typical extraction curve is shown in Figure 2. In this study, serial sections of bovine hoof epidermis were extracted in potassium phosphate buffer at pH 7.0 for 15 min and stained with Harris's hematoxylin. All parameters were kept constant except for molarity, which was varied at 0.1 M intervals for each individual section. The resultant extraction pattern indicated that bovine and rat KH was solubilized in a range of molarities from 0.4 M to 1.6 M. Similar studies done with other salts, including sodium chloride, guanidine hydrochloride, potassium chloride, magnesium chloride, calcium chloride, and ammonium sulfate, indicated that they also solubilized KH and that each salt had a particular extraction pattern [4]. Salts of similar ionic configuration and charge produced extraction patterns that were almost identical, e.g., guanidine hydrochloride, potassium chloride, and sodium chloride were similar, as were calcium and magnesium chloride [4]. Certain salts, e.g., potassium phosphate and ammonium sulfate, did not extract KH at high molarities, and urea did not extract KH at any molarity [4].

To further demonstrate the extraction of KH by salt solutions, we minced tissue into small cubes prior to extraction [2–4]. Histochemically and ultrastructurally, we found that KH was solubilized and extracted from the tissue cubes also. Observations with the electron microscope indicated that tonofilaments were not solubilized by the salt-extraction procedures. After concluding that KH was easily solubilized and therefore could be subjected to more classical isolation methods, we discontinued the cell fractionation techniques.

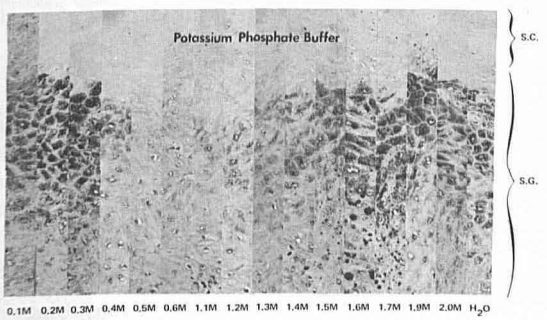


FIG. 2. Composite of serial sections of cattle hoof epidermis which have been extracted in potassium phosphate buffer (pH 7.0) for 15 min at 37°C. Only molarity (M) was varied as indicated. Dark-staining particles are keratohyaline granules. *S.C.*, stratum corneum; *S.G.*, stratum granulosum; H_2O, water control (Harris's hematoxylin; × 75).

IN VITRO AGGREGATION

Given the fact that KH can be solubilized by salt solutions as a function of molarity, can these solubility characteristics be used to isolate KH? It seemed reasonable to assume that KH would be insoluble at concentrations which did not solubilize it. For example, in examining Figure 2, one would assume that KH would be solubilized at a salt concentration of 1.0 M and would precipitate out of solution when the salt concentration was lowered to a value less than 0.4 M. We attempted isolation using this approach by extracting epidermis in 1.0 M potassium phosphate buffer, pH 7.0, for 15 min, clearing the extract by decantation and centrifugation, and dialyzing the extract against distilled water. The clear solution became turbid and by histochemical and ultrastructural examination, we found not only that had the presumptive KH come out of solution but that it looked exactly like the in situ KH granule [2–4]. Such aggregation or self-assembly may result in purification and is usually termed in vitro aggregation or reconstitution [2–4, 6].

The process has been used to purify viruses, collagen, myosin, and many other substances [3]. Many other proteins and other substances are extracted by the salt solutions, but only bovine KH undergoes in vitro aggregation in this particular system [2,4]. The formation of the ordered particles, called macroaggregates [2,4], is presumably based on a built-in set of bond sites and on a tendency for such molecules to form the most stable bonds during aggregation. This particular phenomenon makes the production and isolation of bovine KH technically easy. All the above-mentioned salts produced similar results, but the potassium phosphate experiments produced the most homogeneous macroaggregates [4,11]. On the other hand, dialysis of 8 M urea extracts produced a gel composed of fibrous structures similar to tonofilaments [11].

What does the in vitro aggregation tell us about bovine KH? Macroaggregation, the formation of large aggregates as a function of salt concentration, is predominantly a noncovalent process [2,4]. The information to form noncovalent bonds is built into the molecules when they are synthesized. We are tempted to assume that similar mechanisms are responsible for what is observed in vivo as KH granules gradually increase in size. The dissociation of KH granules at the level of the stratum corneum may also be salt dependent. Examination of the solubility curves for magnesium chloride or calcium chloride indicates that extremely small concentrations of certain ions (at least as small as 0.05 M in the cases of calcium chloride or magnesium chloride) can dissociate the KH granule [4]. This indicates that physiologic but critical salt concentrations in the cytoplasm of the epidermis may be important in determining certain processes of normal and abnormal keratinization. The final

events of keratinization may be controlled not by enzymes, but rather by the unique primary structures of the proteins themselves.

MORPHOLOGIC IDENTIFICATION OF KERATOHYALIN

We have identified KH [2,4,5] by histochemical, ultrastructural, and immunologic methods because we believe that for the isolated material to be identified as KH it: (1) must be stained with all the known stains for KH (hematoxylin, Congo red, the Pauly reagent, sodium alizarin solfonate, toluidine blue, methyl green–pyronin, and acridine orange), (2) must have ultrastructural properties compatible with KH, and (3) must produce antibodies to KH when injected into a foreign species. Actually, the most important assay is the immunologic since, if a single highly purified antigen evokes antibody to the in situ keratohyaline granule (KHG), it must be a constituent of the granule regardless of histochemical and ultrastructural findings. The simplest case, in fact, would be a single molecular species which fulfills all the above criteria. Bovine KH is just such a species.

For our histochemical studies, macroaggregates were collected on the surface of Millipore filters, fixed, and stained with the 7 histochemical stains mentioned above (Fig. 3). We performed identical studies on nonextracted and extracted tissue [2–4]. The results of these studies showed that the isolated macroaggregates had histochemical properties identical with those of the in situ KHG. Large amounts of KH were missing from the extracted tissue but usually there was a rim of residual KH even after extraction. The individual nature of the macroaggregates was not apparent in paraffin sections because of the method of fixation, but when the macroaggregates were embedded in plastic, individual particles about the same size and shape as the in situ KHG were seen (Fig. 4).

At the ultrastructural level, typical KHGs were observed before extraction (Fig. 5); after extraction, only a residual rim of KH was left in the tissue (Figs. 6,7). The macroaggregates were seen to be particles varying in size and shape with an electron-dense core and a less-dense margin (Figs. 8, 9); no limiting membrane was observed.

IMMUNOLOGIC IDENTIFICATION OF KERATOHYALIN

The macroaggregates are composed of a well-defined group of polymers [2,4,6]. When the solubilized bovine macroaggregates are fractionated, a set of molecules of various sizes with identical properties (amino acid composition, spectra, histochemical staining, antigenicity) are seen. In order to determine the origin of these polymers (which have the same properties as bovine KH), antibodies were raised in rabbits to unfractionated macroaggregates or various groups of purified polymers [5]. Indirect immunofluorescent studies with the resultant antisera showed that cytoplasmic particles of the same size and shape as KHGs were strongly fluorescent (Figs. 10, 11). No other area

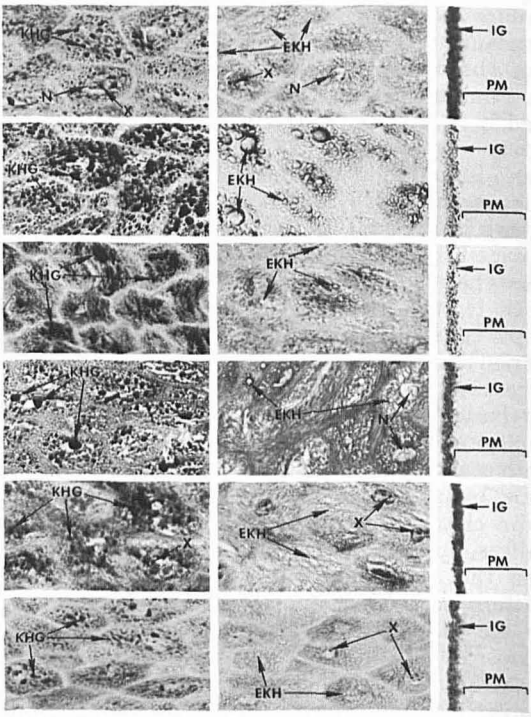


FIG. 3. Paraffin-embedded specimens of nonextracted tissue demonstrating numerous in situ keratohyaline granules (*left column*); tissue extracted in 1.0 M potassium phosphate buffer (pH 7.0) demonstrating extraction of keratohyalin (*middle column*); isolated macroaggregates on surface of porous membranes (*right column*). Specimens are stained with Harris's hematoxylin, Congo red, diazotized sulfonilic acid, sodium alizarin solfonate, toluidine blue, and methyl green–pyronin (*top to bottom*). *KHG*, keratohyaline granule; *N*, nucleus; *X*, nucleolus; *EKH*, area in which keratohyalin has been extracted; *IG*, isolated macroaggregate; *PM*, porous membrane (× 310).

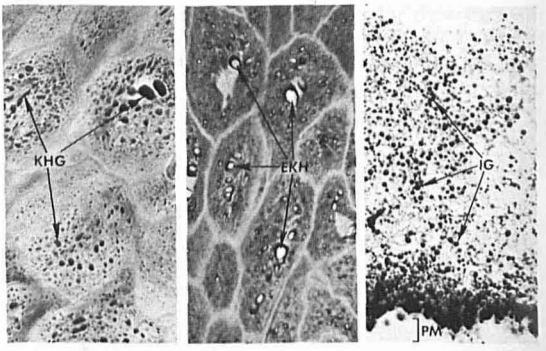


FIG. 4. *Left:* Epon-embedded specimens of nonextracted tissue demonstrating numerous in situ keratohyaline granules (*KHG*). *Center:* Tissue extracted in 1.0 M potassium phosphate buffer (pH 7.0) demonstrating extraction of keratohyalin (*EKH*) and residual rim of KH. *Right:* Isolated macroaggregates (*IG*) on porous membranes (*PM*) demonstrating discrete nature of the macroaggregates and size and shape similar to the in situ KHGs (Azure II and methylene blue; × 530).

was fluorescent except the stratum granulosum, and even here, fluorescence was limited to the in

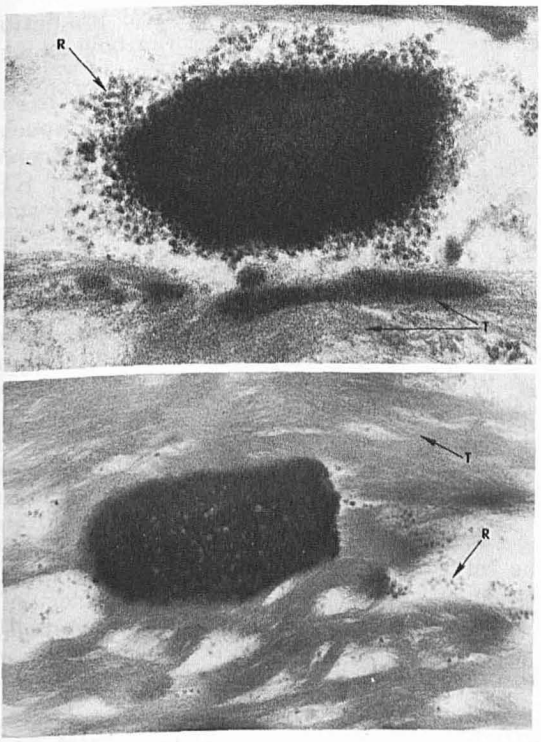


FIG. 5. In situ keratohyaline granules in nonextracted tissue. The *upper* granule lies free in the cytoplasm and is surrounded by ribosomes. The *lower* granule is surrounded by tonofilaments. The granules are amorphous and lack a limiting membrane. *R*, ribosome; *T*, tonofilament (Uranyl acetate and lead citrate; × 24,000).

situ KHGs. Similar results were observed regardless of the origin of the cattle epidermis which was used for indirect immunofluorescence (Figs. 12, 13). The antibody did not cross-react with human, rat, or guinea-pig KH but did have a weak reaction with rabbit KH. The positive immunofluorescent reactions could be completely abolished by previous absorption of sera with either the macroaggregates or any of the purified polymeric species of bovine KH.

FRACTIONATION OF BOVINE KERATOHYALIN

Polyacrylamide gel electrophoresis was used to fractionate the bovine KH species [6,12]. In this technique, a single disc or band usually indicates a single molecular species except in the case of two molecular species of identical size but with different charges. In this case, the two molecules of identical size migrate to different positions on the gel. There are methods of determining whether one is dealing with size or charge isomers [6,12,13], but the gel system which was developed for fractionating bovine KH simplifies this because all such molecules are so charged that regardless of amino acid composition, the charge to mass ratio is constant [6]. This is accomplished by solubilizing the macroaggregates in sodium decyl sulfate, an anionic detergent, which also denatures the molecules. Therefore, in our gel system, a single band indicates a single molecular species and many bands indicate many different species, with certain unique exceptions. When a heterogeneous population of proteins is fractionated in such a system, the fractionated proteins are distributed on the gel without any geometric relationship to each other. If, however, the proteins are arranged on the gel in a particular geometric relationship, other conclusions can be drawn. Specifically, when bovine macroaggregates are solubilized in sodium decyl sulfate and subjected to gel electrophoresis, the bands assume a definite geometric relationship to each other (Fig. 14), demonstrating two features. One, they are arranged logarithmically (band one is farther from band two than band two is from band three and so forth). Two, each of the dark-staining bands travels with a more light-staining member.

The logarithmic distribution of proteins indicates that a set of polymers has been fractionated [4,6]; that is, band one is a single unit, band two is a complex of two units, and so on. The entire set of polymers differs only in size. This can be proved by various methods. For example, if the molecular

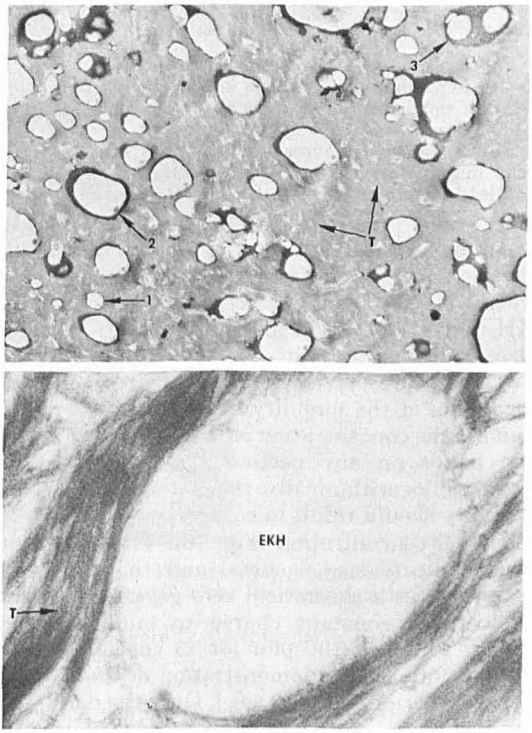


FIG. 6. Epidermis extracted in 1.0 M potassium phosphate buffer (pH 7.0). Keratohyalin (KH) has undergone various degrees of extraction ranging from complete to minimal. *1*, area of complete extraction; *2*, area of residual rim of KH; *3*, area of minimal extraction; *T*, tonofilament (Uranyl acetate and lead citrate; × 4,100).

FIG. 7. Area in which keratohyalin has been completely extracted demonstrating a vacuole (*EKH*) surrounded by tonofilaments (*T*) (Uranyl acetate and lead citrate; × 23,500).

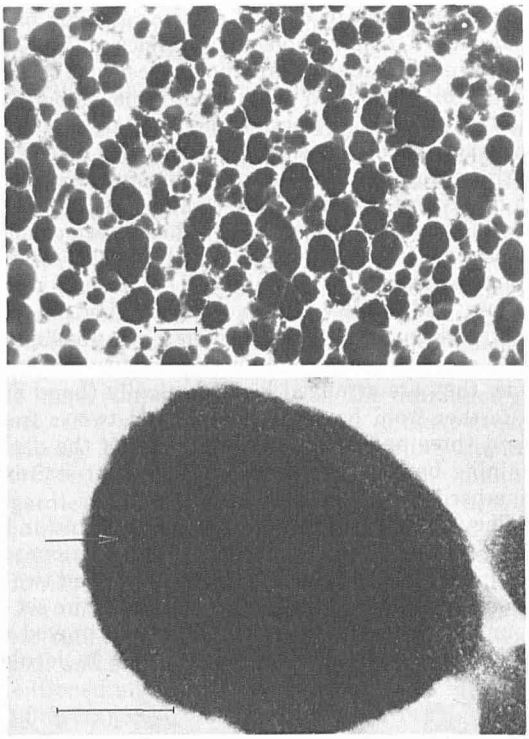


FIG. 8. Isolated macroaggregates which are similar to in situ keratohyaline granules in size and shape and are discrete particles (Uranyl acetate and lead citrate; × 4,100). Bar = 1 μ.

FIG. 9. Isolated macroaggregates with a more prominent margin (*arrow*) and a dense core (Uranyl acetate and lead citrate; × 23,500). Bar = 1 μ.

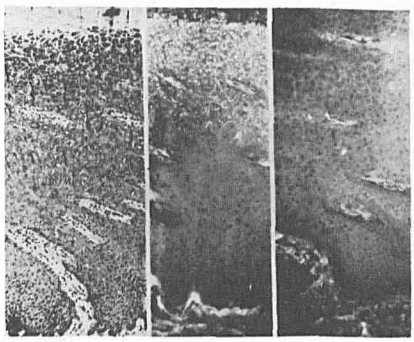
weights of all the individual bands are calculated, each species differs from the next by a constant weight of 16,900 [6]. Moreover, if the solubilized KH is fractionated in many gels of different pore size, a Ferguson plot can be made of all the proteins [6,12,14] (Fig 15). In such a plot, the logarithm of the mobility of each band is plotted against the concentration of the gel (Fig. 16). Since the bands on any particular gel appear to be arranged logarithmically, the plot of the log of their mobility should result in equidistant points at any given gel concentration (Fig. 16). The nonparallel lines indicate size isomers, and the common *y* intercept (at a theoretical zero gel concentration) indicates a constant charge to mass ratio [6]. Figure 16 shows the plot for 13 species and was done before a later demonstration of the 20-member polymeric species [2,4,6,]. Only the dark-staining members of the set have been plotted for clarity [6].

Further proof of homology in bovine KH has been the identical amino acid composition of the various polymers [4], their identical histochemical staining properties [4], identical immunologic properties [5], and the demonstration that the larger polymeric species dissociate slightly into smaller polymeric species [4]. Another proof of homology arises from the theoretical prediction that a set of polymers, like those in bovine KH, will distribute logarithmically [6].

The significance of the doublet pattern is not known, but it has been observed in other special cases [15, 16] and probably represents the presence of conformational isomers. In bovine KH, we see a group of 20 size isomers and 19 conformational isomers.

AMINO ACID ANALYSES AND OTHER BIOCHEMICAL STUDIES

The unfractionated macroaggregates, as well as various polymeric species, were subjected to amino acid analyses [4]. All samples gave similar analyses, and the residues per monomer are shown in the Table. The molecular weight of bovine KH by amino acid analyses is 14,955 [6]. The monomer has 116 residues [4,6]. Serine, glycine, glutamic acid, arginine, and histidine make up about 78% of the total amino acids [4]. Serine constitutes 27% of the total [4]. When 8% is added to the above molecular weight of 14,955 (8% of the molecule is ribonucleic acid; see below), the calculated molecular weight is 16,150, which is in good agreement with the molecular weight of 16,900 as determined



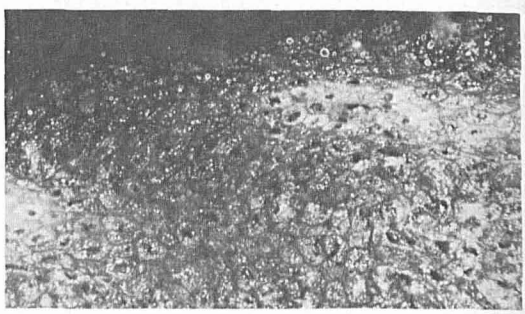


FIG. 10. Methanol-fixed, paraffin-embedded, full-thickness bovine hoof sections. *Left:* Keratohyaline granules (KHGs) stained with Harris's hematoxylin. *Center:* Indirect immunofluorescence with specific antisera which demonstrates particulate fluorescence only over KHGs. *Right:* Treatment with control serum reveals only dermal autofluorescence (× 260).

FIG. 11. View of upper stratum granulosum and lower stratum corneum of bovine hoof treated with specific antiserum. There is fluorescence of the keratohyaline granules and none over the intercellular area, nuclei, and stratum corneum (× 310).

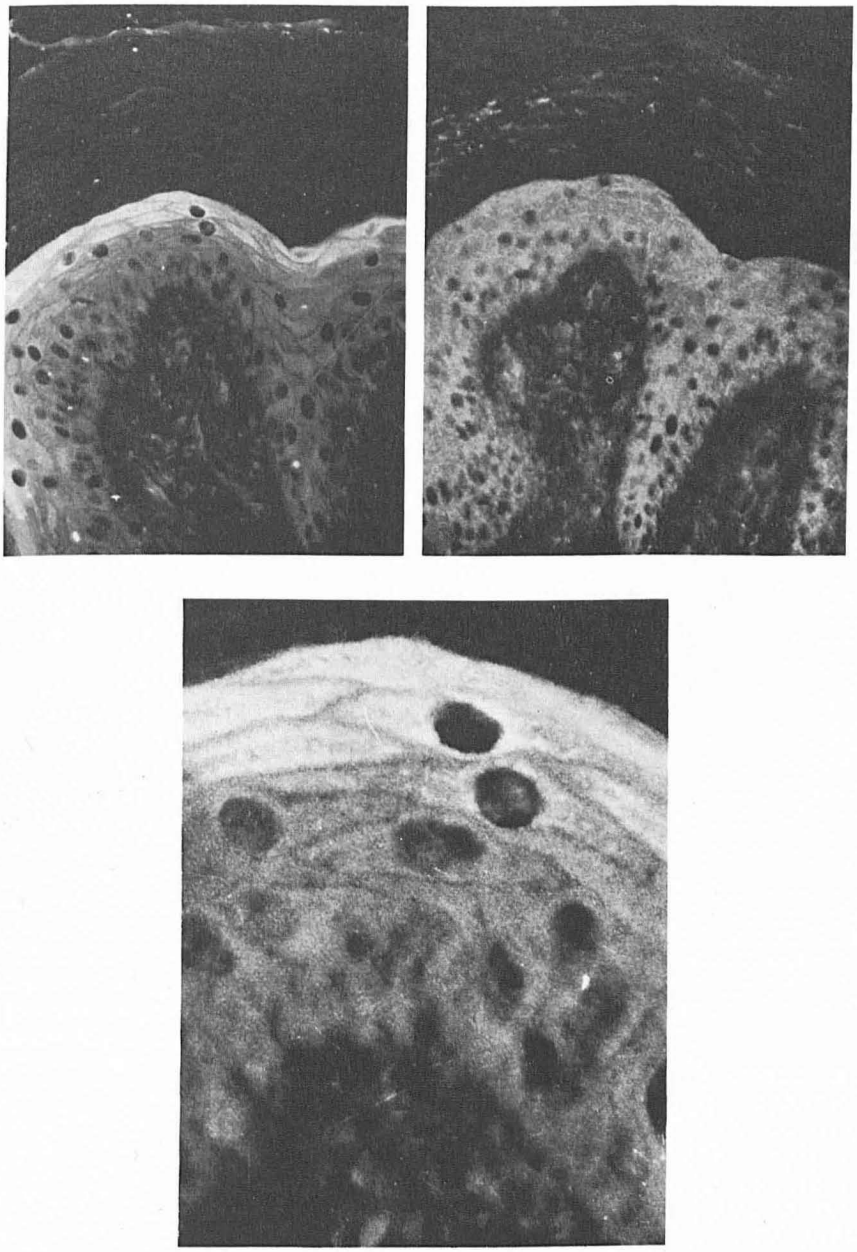


FIG. 12. Bovine abdominal epidermis treated with specific antiserum. The stratum granulosum of this area is thinner than that of the hoof area. Particulate fluorescence is not seen because of the low magnification (Left section, × 256. Right section (control), × 256).

FIG. 13. High-power view of bovine and abdominal epidermis seen in Figure 12, showing particulate fluorescence (× 1,020).

by disc gel electophoresis.

When solubilized KH was analyzed spectrophotometrically, it had a peak of 259 nm (Fig. 17), compatible with the presence of ribonucleic acid [4]. According to the orcinol reaction, about 8% of the macroaggregates was ribonucleic acid [4].

AUTORADIOGRAPHIC STUDIES

To study the labeling pattern of bovine KH, we have used a short-term in vitro culture system [7] in which the synthesis of KH can be modified by the addition of various components to the tissue media. If tritiated histidine is added to the media and incubated for 6 hr, the entire group of KH polymers is labeled. If the incubation period is shortened to only 15 min, only the small polymers are labeled. If puromycin, an antimetabolite which interferes with new protein synthesis at the level of translation, is added to the media before the addition of tritiated histidine, only unlabeled KH

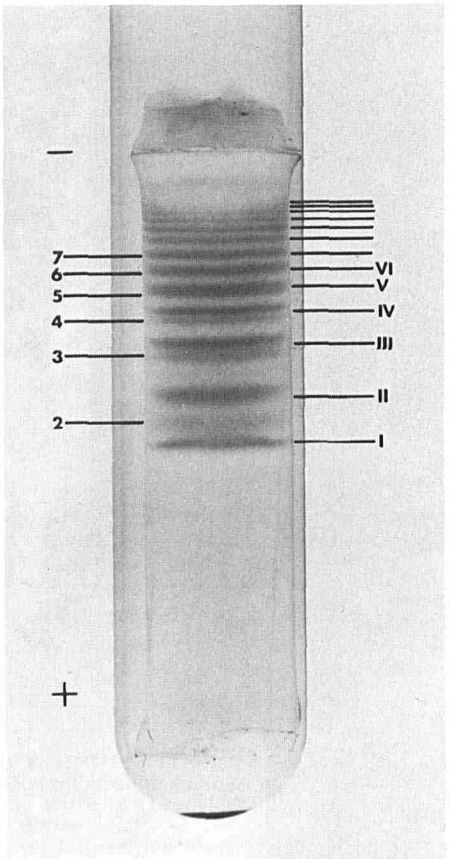


FIG. 14. Fractionation of purified ologomers on a 9% polyacrylamide gel stained with amido black. The first 13 bands are distributed in a geometric series and are composed of doublets in which the slower-moving member of each doublet is more darkly stained (*roman numerals*) than the faster-moving member (*arabic numerals*).

is isolated. If, however, puromycin is added *after* the labeled histidine has been added to the media, it does not interfere with the polymerization mechanism. This suggests that polymerization occurs after release from the ribosomes, probably by an enzyme present in the cytoplasm.

Additional studies using various tritiated amino acids corroborated what others had already shown [17–20], namely, that serine, arginine, glycine, and histidine were incorporated into KH at a faster rate than other amino acids [7].

CORRELATIONS

Reaven and Cox, using the Pauly stain, made tentative observations that KH might contain large quantities of histidine [21,22]. Later, they evaluated certain factors which could modify the amount of Pauly-positive material in the epidermis [23]. They observed that when phosphate buffer at pH 9.0, 0.5 M was used to extract the tissue, it caused a decreased amount of staining, and they concluded that this was due to activation of enzymes which digested the Pauly-positive material. They did not see any decrease when phosphate buffer was used at pH 7.0. In retrospect, our interpretation of their data suggested that they were, in fact, observing KH extraction by their salt solutions.

Autoradiographic studies in rats by Fukuyama, Nakamura, and Bernstein [17], Fukuyama and Epstein [18,19], and by Cox and Reaven [20] further demonstrated that four amino acids were concentrated into KHGs: serine, glycine, arginine, and histidine. These are the amino acids present in the largest quantities in bovine KH. Such studies also suggest that rat KH should be rich in these amino acids, and, in fact, Sibrack, Gray, and Bernstein have recently published such data [24]. The correlation between autoradiographic studies and amino acid analyses also suggests that KH is

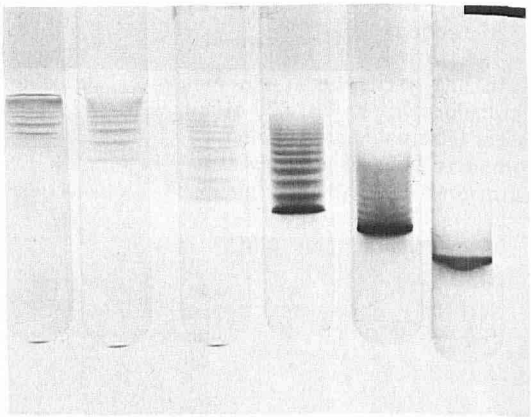


FIG. 15. Fractionation of purified ologomers on 15%, 12%, 9%, 6%, 4%, and 3% polyacrylamide gels stained with amido black (*left to right*).

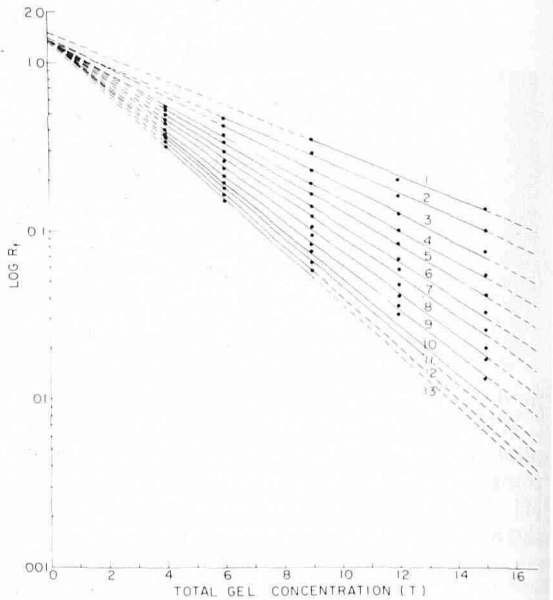


FIG. 16. Log R_f (mobility) versus total gel concentration for each of the major bands in Figure 15.

TABLE. *Amino acid composition of bovine keratohyalin*

Amino acid	Mean[a] ± Standard deviation
Aspartic acid	7.9 ± 0.3
Threonine[b]	7.8 ± 0.3
Serine[b]	31.7 ± 1.0
Glutamic acid	14.8 ± 0.4
Proline	1.0 ± 0.4
Glycine	18.3 ± 0.5
Alanine	3.0 ± 0.4
Half-cystine[c]	1.1 ± 0.4
Valine	0
Methionine	0
Isoleucine	0
Leucine	1.3 ± 0.3
Tyrosine[b]	1.1 ± 0.3
Phenylalanine	0
Lysine	2.1 ± 0.6
Histidine	10.8 ± 0.6
Arginine	14.7 ± 0.5
Tryptophan[d]	0

[a] Residues per subunit (monomer) determined by analyses of bovine monomer, various polymers, and macroaggregates [4]. A value of zero indicates less than 0.3 residue.

[b] Corrected for losses during hydrolyses [4].

[c] Sum of cystine and cysteic acid [4].

[d] Determined colorimetrically [4].

composed predominantly, if not entirely, of such an amino acid-containing protein because two different proteins composed of different amino acids would not permit specific labeling to the point that amino acid composition could be predicted [4].

Other histochemical and ribonuclease digestion studies indicated that ribonucleic acid is complexed to KH [25–28]. Three stains for RNA also stain KH: toluidine blue, methyl green–pyronin, and acridine orange. After digestion with ribonuclease, KH no longer demonstrates such staining, but hematoxylin, Congo red, sodium alizarin solfonate, and the Pauly stain continue to stain the granules. The spectrum of solubilized KH, as well as the evidence for RNA (indicated by the positive orcinol reaction), seems to suggest the presence of RNA. We have not isolated ribonucleotides and regard these data as suggestive, not confirmatory.

COMMENTS

In 1966 Hooper and Bernstein [29] isolated a "histidine-rich" protein from rat epidermis; this topic has been recently reviewed [30]. These studies were fundamental to our own studies since we took Bernstein et al's data to indicate that KH could be solubilized. Bernstein's laboratory has recently applied our high salt extraction and in vitro aggregation methods to their study of rat KH [24] and isolated rat macroaggregates with properties similar to the in situ rat KHG. Further analyses of this material indicate that 50% of the isolated macroaggregate is composed of a protein, 85% of which is composed of glutamic acid, glycine, serine, arginine, alanine, threonine, and histidine, and 7.5% of RNA. Although the amino acid analyses of the rat KH differ significantly from that of bovine KH, there are certain correlations. Polyacrylamide gel electrophoresis of rat KH, however, resulted in a heterogeneous fractionation pattern which is also significantly different from that of bovine KH.

Hypotheses

Two general hypotheses can be proposed at this point: one relates to molecular models, the other to questions of KH function at a more biologic level. Before any intelligent functional models can be developed, however, the questions about molecular models will have to be further explored.

All evidence to date suggests that bovine KH is a self-aggregating substance. Even at the level of the light microscope, one sees evidence of aggregation and then complete dispersal at the junction of the stratum granulosum and stratum corneum. Essential to an understanding of certain questions which need to be explored is our interpretation of polymerization versus macroaggregation. We consider macroaggregation to be a noncovalent proc-

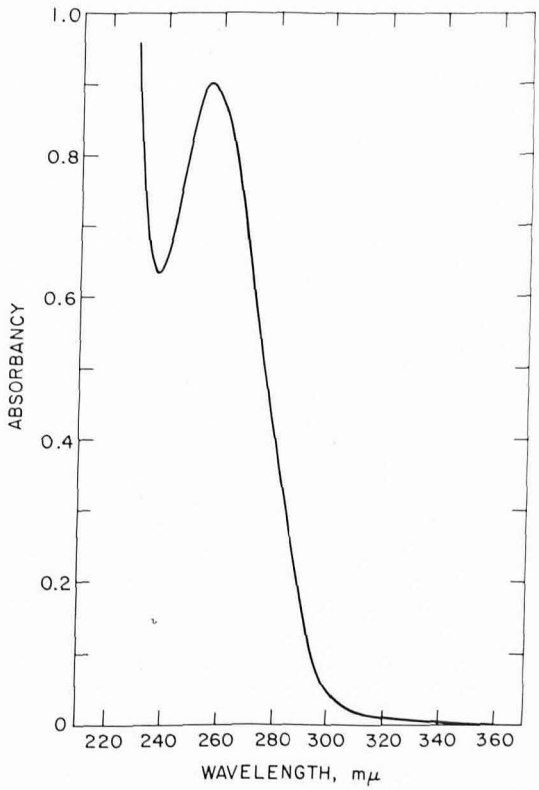


FIG. 17. Spectrum of purified ologomers in 0.02 M sodium decyl sulfate. Maximum absorbancy at 257 nm (protein, 140 μg/ml).

ess because it can be accomplished merely by varying the salt concentration. On the other hand, polymerization, i.e., the formation of the various species that are seen on gel electrophoresis and that compose the macroaggregate, appears to be covalent because we have failed to depolymerize the protein under many conditions that have been used to dissociate noncovalent bonds. Macroaggregation might be considered to be identical to the formation of KHGs in situ, as seen with the light microscope, whereas polymerization is a molecular event, measured by various biochemical techniques.

Before we can make any further progress in understanding KH, we must answer some of the following questions. What is the event which initiates KH synthesis? What determines the number of polymeric units to be synthesized? Are there specific polymerization enzymes? What biochemical cross-link is used for polymerization? All of these questions relate to events which probably occur before macroaggregation. But we must also investigate the following questions which relate to macroaggregation and dispersal. What determines the initiation of macroaggregation? Is macroaggregation in vitro similar to macroaggregation in vivo? What initiates dispersal (dismacroaggregation)? Is dismacroaggregation a noncovalent process or are specific depolymerizing enzymes required? What is the eventual fate of the dispersed KH?

The most frustrating area of all at this time is the lack of any critical data to suggest a function of KH. However, as the molecular models are explored, certain probes will become available which will enable us to study the function of this intriguing epidermal substance.

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